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The *asiA* Gene of Bacteriophage T4 Codes for the Anti-σ^{70} Protein

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The anti-σ^{70} factor of bacteriophage T4 is a 10-kDa (10K) protein which inhibits the σ^{70}-directed initiation of transcription by *Escherichia coli* RNA polymerase holoenzyme. We have partially purified the anti-σ^{70} factor and obtained the sequence of a C-terminal peptide of this protein. Using reverse genetics, we have identified, at the end of the lysis gene *t* and downstream of an as yet unassigned phage T4 early promoter, an open reading frame encoding a 90-amino-acid protein with a predicted molecular weight of 10,590. This protein has been overproduced in a phage T7 expression system and partially purified. It shows a strong inhibitory activity towards σ^{70}-directed transcription (by RNA polymerase holoenzyme), whereas it has no significant effect on σ^{70}-independent transcription (by RNA polymerase core enzyme). At high ionic strength, this inhibition is fully antagonized by the neutral detergent Triton X-100. Our results corroborate the initial observations on the properties of the phage T4 10K anti-σ^{70} factor, and we therefore propose that the gene which we call *asiA*, identified in the present study, corresponds to the gene encoding this T4 transcriptional inhibitor.

During infection of *Escherichia coli* by phage T4, a large part of the program of viral gene expression is regulated at the transcriptional level. The host's RNA polymerase transcribes the 200 or so viral genes from different classes of T4-specific promoters (23). Some of these classes are recognized only after T4-coded functions modify this enzyme's specificity. The α subunits of RNA polymerase are covalently modified by ADP-ribosylation, and the enzyme binds a series of phage-coded proteins (13). Among these, the products of genes *33*, *45*, and *55* are required for late transcription (12). In particular, gp 55 (16) redirects RNA polymerase transcription initiation from T4 late promoters by replacing the *E. coli* σ^{70} subunit. The product of gene *rpbA* (45) strongly binds to RNA polymerase core. A smaller RNA polymerase-binding gene product, the 10-kDa (10K) protein, copurifies with σ^{70} on phosphocellulose and inhibits σ^{70}-directed transcription initiation by *E. coli* RNA polymerase holoenzyme (36). It is believed that the interaction of the T4 10K protein with bacterial σ^{70} weakens σ^{70}-core interaction; this, in turn, would allow gp 55 to successfully compete for core enzyme during T4 development (12). The 10K protein found in lysates from T4-infected cells strongly binds to RNA polymerase agarose affinity columns (27). Starting from this observation, we have previously used a biochemical approach to identify the gene encoding the T4 10K protein (25). Such affinity columns were utilized to chromatographically analyze the radioactive proteins synthesized in vitro by a coupled transcription-translation system primed with fragments of T4 DNA. This attempt yielded the unexpected result that there seemed to exist in the phage T4 chromosome two widely separated regions which coded for 10-kDa proteins having a high affinity for RNA polymerase. Since this technique was unsatisfactory for screening DNA fragments with the size of individual genes, we have changed our approach. We have sequenced a short peptide isolated from the 10K protein and have used reverse genetics to locate the corresponding gene. In the present article, we report the identification of a gene encoding the T4 10K protein, its nucleotide sequence, and the anti-σ^{70} properties of its overexpressed product. We propose to name this gene *asiA*, for anti-sigma 70 (or Audrey Stevens' inhibitor).

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MATERIALS AND METHODS

Materials. DEAE-Sephadex A50 was from Pharmacia. Bio-Gel A-0.5m and Bio-Rex 70 were from Bio-Rad. DEAE-cellulose DE-52 was from Whatman. Restriction endonucleases were from Boehringer and Bethesda Research Laboratories, Inc. Avian myeloblastosis virus reverse transcriptase was from Boehringer. T4 DNA ligase was from Promega and poly(dA-dT) was from Sigma.

Bacteria, phages, and plasmids. *E. coli* B^E (*sup*) was our standard strain for phage infection and physiological studies. It was also used as the source of RNA polymerase for enzyme purification. T4 D^+ was our wild-type bacteriophage strain. Phagemid Bluescript pBSKII (+) was used for cloning the *asiA* gene and was obtained from Stratagene. M13mp18 cloning vector (47) was used for site-directed mutagenesis and was purchased from Boehringer. *E. coli* JM101 (47) and XL1-Blue (15) were used for transformations and were obtained from Stratagene. *E. coli* RZ1032 (*dut ung*) was used to prepare uracil-containing templates for site-directed mutagenesis (18). Stocks of the lambda CE6 derivative carrying phage T7 gene *1* (40) were prepared in *E. coli* strains YMC (*sup*$^+$) and ED8689 (r^- *sup*) and were kindly donated by M. Uzan.

Partial purification of the T4 10K protein (gp asiA). T4-infected bacteria used for the isolation of the 10K protein were a generous gift from E. P. Geiduschek's laboratory. The cells were *E. coli* DG 156 ($RNase^-$) grown at 37°C in Luria-Bertani (LB) medium and infected at a multiplicity of infection of 11 with phage T4 *regA 45 am*. The cells were

harvested 12 min after infection, frozen, and stored at −70°C. The partial purification procedure was based on the σ^{70}-binding properties of gp asiA and on the σ^{70}-binding capacity of unmodified RNA polymerase core enzyme. Unmodified RNA polymerase was thus used as a carrier for gp asiA (36). The starting material was a mixture of 170 g of frozen *E. coli* B^E cells and 30 g of T4-infected bacteria. In addition to this amount of cells, and in order to radioactively label the 10K protein, 300 ml of *E. coli* B^E was grown at 37°C in M9 medium without Casamino Acids to 7×10^8 cells per ml and UV irradiated; this culture was then infected with $T4^+$ (multiplicity of infection = 7), and the proteins were labeled with L-[^{35}S]methionine from 1 to 8 min after infection (25). Labeled phage proteins were obtained after lysis by lysozyme and DNase treatment followed by centrifugation of the cell debris (43).

The unlabeled 200 g of mixed bacteria was submitted to the lysis procedure and the Polymin P precipitation described by Burgess and Jendrisak (3). The RNA polymerase activity was fractionated with ammonium sulfate (2). At this point, the radioactive lysate of T4-infected cells was added to the unlabeled lysate, and RNA polymerase was purified from this mixture as described elsewhere (9) with the following modifications: the first chromatography on 0.5-m agarose was performed at a low salt concentration (50 mM KCl), and the DEAE-Sephadex step was followed by two successive chromatographies on a 0.5-m agarose column in the presence of 1 M KCl. The radioactive holoenzyme preparation was separated into core polymerase and σ^{70}-containing fractions on a Bio-Rex 70 column (21). The flowthrough fractions containing σ^{70} and the radiolabeled 10K protein were concentrated on a short DEAE-cellulose column, whereas the core polymerase was eluted from the Bio-Rex 70 column as described elsewhere (21). The proteins were analyzed by electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide gels composed of a discontinuous gradient of 5 to 17.5% acrylamide (25).

Isolation and sequence analysis of the 10K protein. The radioactive flowthrough fractions of the Bio-Rex 70 column were subjected to preparative electrophoresis on SDS-polyacrylamide gels. The gel band containing the radioactive 10K protein was excised. The protein was electroeluted and purified by reverse-phase high-pressure liquid chromatography on a C4-300 column (4.6 by 30 mm). Approximately 130 pmol of radioactive protein was digested by trypsin (treated with L-1-tosylamido-2-phenylethyl-chloromethyl ketone) at a final enzyme-to-substrate ratio of 1:40 (wt/wt) for 2 h at 37°C. The resulting digest was fractionated by reverse-phase high-pressure liquid chromatography on a Brownlee RP-300 octyl-silica column (2.1 by 100 mm), and 10% of the injected radioactivity was found in one peptide peak. This material was analyzed by automated Edman degradation in a 470-A gas-liquid phase Sequencer (Applied Biosystems). Phenylthiohydantoin derivatives were analyzed with a 120-A phenylthiohydantoin amino acid analyzer.

T4 phage infection and RNA purification. *E. coli* B^E was grown at 30°C in MOPS (morpholinepropanesulfonic acid)-Tricine minimal medium (24), supplemented with 1% L-Casamino Acids, to a density of 5×10^8 cells per ml, and the bacteria were infected by T4 phages at a multiplicity of 7. At given time points, aliquots of the infected cells were added to an equal volume of lysis buffer containing 1.66% SDS, 0.5 M sodium acetate was adjusted to pH 4.8, and 6 mM EDTA was placed in a boiling water bath for 1 to 2 min. RNA was purified as previously described (42).

Primer extension and RNA sequencing reactions. The synthetic 24-mer oligonucleotide 5′TTTGTTCGTATACATC TCTAGATA3′, complementary to positions 343 to 320 (see Fig. 2), was ^{32}P end labeled and hybridized to RNA isolated 5 min after T4 D^+ infections of *E. coli* at 30°C. RNA was analyzed by primer extension and sequenced by the dideoxynucleotide chain termination method according to the protocols described by Uzan et al. (42).

Cloning of gene *asiA*. The putative open reading frame (ORF) of gene *asiA* was inserted in the multiple cloning site of the Bluescript pBSKII (+) vector under the control of a phage T7 RNA polymerase promoter (40). The cloning procedure was as follows. Cytosine-substituted T4 DNA, purified from phage T4 dC strain *alc* w7 as previously described (25), was cleaved with *Bgl*I and *Kpn*I. The digestion products were subjected to electrophoresis on a 0.5% agarose gel. A 6.95-kb *Bgl*I-*Kpn*I fragment harboring the region of lysis gene *t* (22) between coordinates 153.35 and 160.3 kb on the phage T4 genomic map was recovered from agarose by using Geneclean (Bio 101). Two oligonucleotides were designed to prime the polymerase chain reaction used to amplify a 307-bp fragment from the purified 6.95-kb *Bgl*I-*Kpn*I restriction fragment. A restriction site was included in the sequence of each primer to facilitate subsequent cloning of polymerase chain reaction products. The nucleotide positions refer to the numbering used in Fig. 2. Primer 1 was a 63-mer extending from nucleotides 40 to 102. It contained four mismatches at positions 50 to 54, generating a *Hin*dIII site upstream of the N-terminal end of ORF *asiA*. Primer 2 was a 60-mer complementary to nucleotides 377 to 318. It contained three mismatches at positions 360, 359, and 357, generating a *Pst*I site downstream of the *asiA* ORF stop codon. Polymerase chain reaction was performed with a Hybaid thermal reactor. The reaction mixture contained 500 ng of template DNA, 100 pmol of each primer, the four deoxynucleoside triphosphates at 0.2 mM each, and 2.5 U of *Taq* DNA polymerase in the buffer recommended by the manufacturer. Prior to the addition of the enzyme, the reaction mixture was submitted to a 5-min denaturation at 95°C. After completion of 30 cycles, the reaction mixture was analyzed by electrophoresis on a 1.5% agarose gel and the amplified product was purified by phenol-chloroform extraction as described in reference 30. The product was then digested with *Hin*dIII and *Pst*I and cloned into pBSKII (+) vector which had previously been digested with the same two enzymes. Competent *E. coli* JM101 cells were transformed with the product of the ligation reaction and were plated on IPTG (isopropyl-β-D-thiogalactopyranoside)–X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) LB plates containing 150 μg of ampicillin per ml. Several white colonies were isolated and grown; plasmid DNA was extracted and purified by the rapid method of Saunders and Burke (32). The presence of the 307-bp insert was verified by 2% agarose gel electrophoresis after digestion of plasmid DNA with *Hin*dIII and *Pst*I. This plasmid was designated pBAS-W1.

Site-directed mutagenesis of the ribosome binding site. The GTGG Shine-Dalgarno sequence of the *asiA* gene was replaced by a GGAG sequence 9 nucleotides upstream of the ATG codon. This was effected by site-directed mutagenesis to improve the translation efficiency of the cloned *asiA* gene. A 252-bp *Sal*I-*Eco*RI fragment from pBAS-W1 was subcloned between the *Sal*I and *Eco*RI sites of the M13mp18 replicative form. A 46-mer synthetic oligonucleotide was used to mutagenize the *asiA* gene Shine-Dalgarno sequence by introducing point mutations A(61)→G and T(63)→A. Uracil-containing viral DNA templates were prepared from

E. coli RZ1032, and the methods described by Kunkel et al. (18) were used for all the in vitro mutagenesis experiments. The *SalI-EcoRI* fragment was then reintroduced into its initial position in pBAS-W1, thus generating a complete copy of *asiA* with a mutated ribosome binding site. This plasmid will be referred to as pBAS-M1.

DNA purification and sequence analysis. Plasmids pBAS-W1 and pBAS-M1 were amplified in *E. coli* XL1-Blue grown in the presence of 100 μg of ampicillin per ml. Plasmid DNA was purified by CsCl gradient centrifugation, and the nucleotide sequences of the cloned inserts on both strands were determined by the dideoxynucleotide chain termination method (31). Sequenase version 2.0 (U.S. Biochemical Corp.) was primed with the M13 and T3 sequencing primers (Stratagene).

Overproduction and partial purification of gp asiA. *E. coli* JM101 competent cells were transformed with plasmid pBAS-M1 and plated in the presence of 200 μg of ampicillin per ml. Single colonies were grown overnight in 40 ml of LB medium containing 200 μg of ampicillin per ml, 10 mM $MgSO_4$, and 0.2% maltose. One overnight culture was used to inoculate 4 liters of LB medium containing the same additives. The bacteria were grown at 37°C to 10^8 cells per ml and infected with a fresh preparation of lambda CE6 (40) at a multiplicity of 10. Ten minutes after infection, rifampin was added to 400 ml of infected cells at a final concentration of 8 μg/ml. Four minutes later, L-[^{35}S]methionine (NEN) was added to these cells at 10 μCi/ml of cells. Incorporation was stopped 30 min later by addition of L-methionine at a final concentration of 0.5 mM. The bacteria were harvested by centrifugation at 4°C. Seven grams of overproducing bacteria were thus obtained and kept in liquid nitrogen until use. These cells were mixed with an equal amount of *E. coli* B^E, and the bacteria were submitted to the lysis procedure of Burgess and Jendrisak (3). All operations were carried out at 4°C. RNA polymerase activity and trichloroacetic acid-insoluble L-[^{35}S]methionine radioactivity were monitored during the purification. The low-speed supernatant was made 0.2 M in NaCl, and nucleic acids and proteins were precipitated with 0.45% Polymin P (3). The insoluble material was carefully ground with a tissue homogenizer, and the proteins were extracted with 0.5 M NaCl. The insoluble material was separated from the supernatant by centrifugation, and proteins were extracted by homogenizing the insoluble material in the presence of 1 M NaCl. The 0.5 and 1.0 M NaCl supernatants were found to contain comparable amounts of radioactivity and RNA polymerase activity. They were pooled and concentrated by 60% ammonium sulfate precipitation. The radioactive precipitate was redissolved in buffer A described by Burgess (2) plus 50 mM KCl and dialyzed against this solution. The dialysate was applied to a 350-ml column (3.2 by 44 cm) of Ultrogel AcA 44 (Industrie Biologique Française) equilibrated with buffer A plus 50 mM KCl. Fractions (5 ml each) were collected and analyzed for their content in RNA polymerase, in radioactive proteins, and in anti-σ^{70} activity. The fractions containing the overproduced protein of gene *asiA* were kept at −20° in a storage buffer containing 50% glycerol (2). Protein concentrations were determined by using the Bio-Rad protein assay reagent and lysozyme as a standard.

RNA polymerase partial purification and activity assay. RNA polymerase was partially purified from *E. coli* B^E by a combination of published methods. The cells were disrupted and lysed, and RNA polymerase was obtained after precipitation with and elution from Polymin P (3). The enzyme was then purified as described by de Franciscis and Brody (9) and stored at −20°C in the 50% glycerol buffer described by Burgess (2). Polymerase activity was determined by incubating diluted aliquots of the enzyme in the presence of the appropriate DNA template [100 μg of calf thymus DNA, 10 μg of T4 DNA, or 10 μg of poly(dA-dT)] for 10 min at 37°C in a 500-μl reaction mixture containing 100 mM Tris-HCl (pH 7.9), 200 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM each ATP, CTP, and GTP, and 0.1 mM [5-^{3}H]UTP (45 Ci/mol). Incorporation of UTP was determined from trichloroacetic acid-precipitable counts. With this assay, one activity unit of RNA polymerase incorporates 1 nmol of UTP in a 10-min reaction.

Computer analyses. Computer analyses of nucleic acid and protein sequences were performed by using the facilities offered by the Base Informatique sur les Séquences d'Acides Nucléiques pour les Chercheurs Européens at the Centre InterUniversitaire d'Informatique à Orientation Biomédicale, Paris, France. In some cases, we have used the sequence analysis software package of the University of Wisconsin Genetics computer group (10).

Nucleotide sequence accession number. The sequence of gene *asiA* has been deposited in the GenBank data base under accession no. M99441.

RESULTS

Partial purification of the 10K protein. In the present study, we have used unmodified RNA polymerase of *E. coli* as an affinity carrier to strongly bind the 10K protein of phage T4 (36). This approach to the partial purification of gp asiA was chosen to minimize its progressive loss, observed during the purification of RNA polymerase from T4-infected cells (35). In Fig. 1, we show the autoradiography of an SDS-polyacrylamide gel illustrating the separation of the RNA polymerase-binding proteins coded by phage T4 after Bio-Rex 70 chromatography. The gp asiA is essentially found in the flowthrough fractions together with massive amounts of the unlabeled σ^{70} protein (Fig. 1, lanes 3 and 4). The core enzyme is retained by the column and binds (Fig. 1, lanes 5 and 6) the strongly labeled gp rpbA (the 15K protein) (45) and gp 55 (28).

Amino acid sequence data and search for a putative ORF. Initial attempts at microsequencing gp asiA, isolated as described in Materials and Methods, were hampered by the extreme insolubility of the protein after electroelution from polyacrylamide gel followed by reverse-phase high-pressure liquid chromatography to remove SDS. Efficient solubilization of the radioactive protein was achieved by trypsinolysis; a radioactive peptide containing tyrosine(s) was purified and was successfully submitted to sequence analysis. An unambiguous element of amino acid sequence, Tyr-Leu-Glu-Met-Tyr-X-Asn, was determined.

Residue X was uncertain. This sequence contained tyrosyl residues, as could be expected from the absorbance of the peptide monitored during its purification, and a methionyl residue accounting for its radioactivity. Computer analysis was used to search, in a data bank containing the available T4 DNA sequences, for a hitherto unassigned nucleotide sequence encoding our short peptide sequence. We found that this peptide sequence can be encoded by a genome region adjacent to lysis gene *t* (22). It is part of the C terminus of a potential protein expressed from the *l* strand of the T4 chromosome, the ORF of which ends with a TAA stop codon, immediately adjacent to the stop codon of gene *t*. The last nine residues encoded by this potential ORF were Arg-Tyr-Leu-Glu-Met-Tyr-Thr-Asn-Lys-STOP.

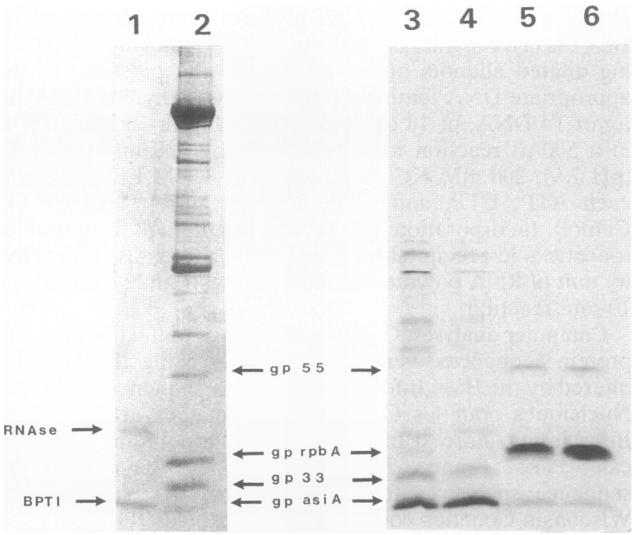

FIG. 1. Chromatographic partition of the 10K protein between σ^{70} and core RNA polymerase. Partial purification of the 10K protein is described in Materials and Methods. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis. Lanes 1 and 2, Coomassie brillant blue staining of the gel; lanes 3 to 6, autoradiograph of this gel. Lanes: 1, reduced and carboxymethylated RNase A (molecular weight = 14,000) and bovine pancreatic trypsin inhibitor (BPTI) (molecular weight = 6,800) protein markers; 2, T4-modified RNA polymerase (generously provided by R. Mailhammer, Max Planck Institut für Biochemie, Martinsried bei München, Germany) (the positions of the T4-coded gp 55, gp rpbA [the 15K protein], gp 33 [the 12K protein], and the 10K protein, gp asiA, are indicated); 3 and 4, flowthrough fractions of the Bio-Rex 70 column further concentrated on DEAE-cellulose (these fractions contained important amounts of unlabeled σ and the labeled gp asiA); 5 and 6, RNA polymerase fractions eluted from the Bio-Rex 70 column by a salt gradient (these fractions contained the labeled T4 proteins gp 55 and gp rpbA).

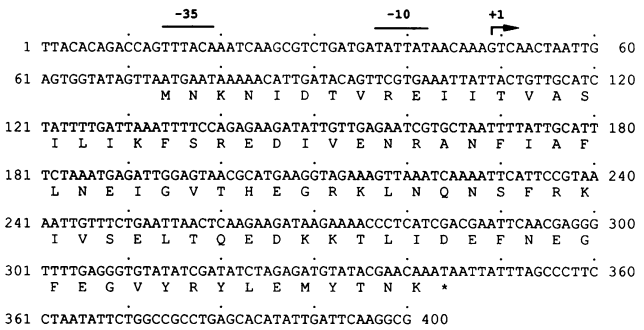

FIG. 2. Nucleotide sequence of gene *asiA*. The first 48 nucleotides of this sequence include the promoter P158.7, identified by Liebig and Rüger (20). The −35 and −10 consensus regions of the promoter are indicated. The arrow at nucleotide 49 indicates the transcriptional start of the gene. The ORF of late gene *t* is coded on the opposing DNA strand, and the sequence complementary to nucleotides 347 to 400 encodes the C-terminal part of gene product *t* (22).

The amino acid sequence contained the five residues of our sequenced peptide preceded by an arginine, as expected for a tryptic peptide, and an asparagine residue, in accordance with our microsequence results. Therefore, there existed a strong probability that the ORF of the 10K anti-σ^{70} gene is contained in the short, and hitherto unsequenced, region of the T4 chromosome lying between the *motA* gene region sequenced by Uzan et al. (41) and the end of lysis gene *t* (22). This probability was reinforced by the presence, some 300 bp upstream of the end of gene *t*, of the early and so far unassigned promoter P158.7, which is one of the 29 strong T4 early promoters cloned and sequenced by Liebig and Rüger (20). Furthermore, Southern blot analysis with a degenerate probe deduced from the amino acid microsequence (according to a T4 codon usage table) was performed. When cytosine-substituted T4 DNA restriction fragments obtained by digestions with several enzymes were probed, the smallest most strongly hybridizing fragment was a 6.95-kb *BglI-KpnI* double-digestion fragment, extending from coordinates 153.35 to 160.3 kb on the T4 physical map and containing the lysis gene *t* region (results not shown).

Taken together, these results led us to investigate the presence of an ORF coding for a 10-kDa protein at the end of the coding sequence of lysis gene *t*.

Identification of gene *asiA*. T4 RNAs isolated 5 min after T4 D^+ infection of *E. coli* cells at 30°C were hybridized to the ^{32}P-labeled 24-mer primer and used as templates for avian myeloblastosis virus reverse transcriptase. We thus identified a major stop of the elongation reaction marking the 5′ end of transcripts extending 300 nucleotides from the primer and initiating at promoter P158.7 (20). These transcripts were entirely sequenced, and this revealed the presence, in a region of high coding probability and downstream from the P158.7 early promoter, of an ORF encoding a protein of 90 amino acids with a predicted molecular weight of 10,590. Reading of the RNA sequence was possible up to the −10 consensus region of the P158.7 promoter because of the presence, at 5 min after T4 D^+ infection, of longer transcripts initiating upstream from this promoter. Minor hard stops corresponding to shorter transcripts were also visible in the primer extension sequencing products. We found (26) that these shorter transcripts are the products of the endonucleolytic cleavage, at an internal GGAG sequence, of the full-length P158.7 transcripts by the endoribonuclease encoded by the T4 *regB* gene (29, 42). Purified plasmid pBAS-W1 DNA harboring the cloned copy of gene *asiA* was used to check the correctness of the gene sequence first obtained by sequencing T4 early transcripts; both DNA strands were sequenced for this check. We show in Fig. 2 the nucleotide sequence of gene *asiA* and the deduced amino acid sequence of its product. We describe below the in vitro anti-σ^{70} properties of the protein overproduced from a cloned copy of this gene. In particular, we show that it behaves essentially like the T4 10K anti-σ^{70} protein identified and purified from T4-infected *E. coli* cells (35–38). We have, therefore, good reasons to believe that we have identified the gene encoding the T4 inhibitor of *E. coli* RNA polymerase holoenzyme. Recently, we have learned that this region of the T4 genome has been independently sequenced by D. Nguyen and J. W. Drake (24a). Their sequence and that shown in Fig. 2 are identical.

Features of the asiA protein. The 90-residue gp asiA contains no proline, cysteine, or tryptophan. Proline has been reported as missing in the 10K anti-σ^{70} protein (34). However, proline, cysteine, and tryptophan are relatively rare residues, and the probabilities of their occurrence are accordingly lower as smaller protein sequences are considered. For example, they are missing in the 67-amino-acid rIIA-1 protein (8) as well as in the 89-amino-acid dsbA protein (11), initially thought to be the 10K anti-σ^{70} protein (14).

In Fig. 1 (lanes 1 to 4) we show that gp asiA migrates at the same level as bovine pancreatic trypsin inhibitor with SS bonds reduced and carboxymethylated, a protein with a molecular weight of 6,500 (7). Anomalous electrophoretic mobilities for proteins with molecular weights lower than approximately 15,000 are generally observed (33, 44). Two T4-coded low-molecular-weight proteins, gp dsbA (11) and gp 33 (46), exhibit significant differences between the molecular weights deduced from their amino acid sequences and the apparent molecular weights corresponding to their electrophoretic mobilities. In the case of gp asiA, the calculated pI is 5.31, a value which reflects its high content in acidic side chains (11 glutamic and 4 aspartic). This acid contribution and the small size of this protein most likely account for its high electrophoretic mobility in our SDS-polyacrylamide gradient gel system (25; also, this work). The sequence of gp asiA was compared with the sequences stored in the available protein data bases. No significant similarities were observed following extensive search in the MIPS protein data base (release no. 31). Analysis of the codon usage in gene *asiA* showed a significant bias in favor of codons used in phage T4 early genes (6) relative to those used in *E. coli* genes (data not shown).

Eight nucleotides upstream from the ATG initiation codon (Fig. 2), we find the sequence GTGG that we think to be the Shine-Dalgarno sequence of gene *asiA*. The spacing between these two translation signals is close to the consensus for the T4-specific ribosome binding sites (7 nucleotides [20]). However, this sequence is markedly different from the optimal GGAG, and it can be presumed that gene *asiA* transcripts are translated with a low efficiency. The ribosome binding properties of the nucleotide sequence around the initiation codon were probed with the W101 matrix developed by Stormo et al. (39). The resulting score of this evaluation fell far below the ranking attributed to efficient ribosome binding sites. In view of the extremely high toxicity of gp asiA (see below), it is likely that its concentration is subject to some kind of strict upper limit in T4-infected cells. Analysis of its regulation warrants further study.

Overexpression of gene *asiA*. Not unexpectedly, the product of gene *asiA* proved to be very toxic when overproduced in *E. coli*. Overexpression was first attempted in *E. coli* BL21(DE3) (40). Even in uninduced cells, the basal level of T7 RNA polymerase was high enough to render transformed cells visibly sick and unable to tolerate plasmid pBAS-W1, harboring the wild-type copy of gene *asiA*. This plasmid appeared to be highly unstable in BL21(DE3) and most often was simply lost. Better results were obtained when phage T7 gene *1* was introduced by infection with lambda CE6 (40) into *E. coli* JM101 carrying plasmid pBAS-W1. The effect of changing the presumed wild-type Shine-Dalgarno sequence GTGG into GGAG was studied. We monitored the time course of gp asiA synthesis after induction of JM101 cells transformed with either pBAS-W1 or pBAS-M1. We show in Fig. 3 the greatly enhanced accumulation of gp asiA when the wild-type sequence (Fig. 3B) has been changed into the much more efficient sequence GGAG (Fig. 3C). The accumulated protein was visualized by radioactive labeling, and the expression of the gene under the exclusive control of T7 promoter and T7 RNA polymerase was made clearer when *E. coli* RNA polymerase was inhibited with rifampin (Fig. 3B and C [time point aliquots are indicated with an R subscript]). The amount of overproduced asiA protein, however, was limited; it was barely detectable upon Coomassie blue staining of the gels. This overproduction system was nevertheless employed to partially purify gp asiA and to analyze its properties.

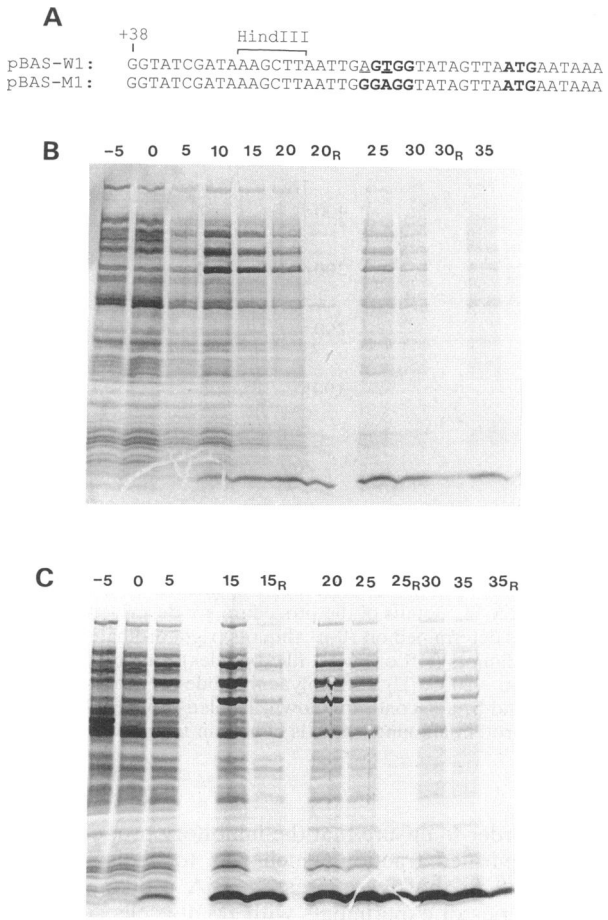

FIG. 3. Overexpression of gp asiA. (A) Nucleotide sequence of the Shine-Dalgarno region for the wild-type gene in pBAS-W1 and the mutagenized version in pBAS-M1. Position +38 is relative to the transcription initiation site from T7 promoter. The *Hin*dIII bracket shows the insertion site of this end of gene *asiA* in vector pBSKII (+). The mutagenized nucleotides are underlined in the wild-type element of sequence. The translation initiation signals (the Shine-Dalgarno sequence and the start codon) are shown in boldface. (B and C) Gene *asiA* overexpression from pBAS-W1 and pBAS-M1, respectively. *E. coli* JM101 cells transformed with pBSKII (+) carrying the cloned copy of gene *asiA* were grown at 37°C in LB medium containing 200 μg of ampicillin per ml, 10 mM $MgSO_4$, and 0.2% maltose. At a concentration of 1.5×10^8 cells per ml, the cultures were infected with lambda CE6 (multiplicity of infection, 10). Immediately before or at the times indicated (in minutes after infection), 2-ml samples were labeled for 5 min with 30 mCi of L-[^{35}S]methionine, and incorporation was stopped by adding 5 mM methionine and 10 mM NaN_3 to each sample. At the time points with an R subscript, rifampin (final concentration, 8 μg/ml) was added to the sample 2 min before labeling. Each sample was centrifuged, and the cells were washed with 1 ml of 50 mM Tris-HCl (pH 8.0), resuspended in 400 μl of sample buffer, and lysed at 100°C for 5 min. Radioactive lysate (60 μl) was loaded on each lane of the SDS-polyacrylamide gradient gel. After Coomassie blue staining and drying, the gels were autoradiographed for 5 days.

Inhibitor properties of overproduced asiA protein. To isolate the product of gene *asiA*, we chose to increase the σ^{70} content of the initial protein mixture by mixing an equal amount of B^E cells with the induced and radiolabeled JM101

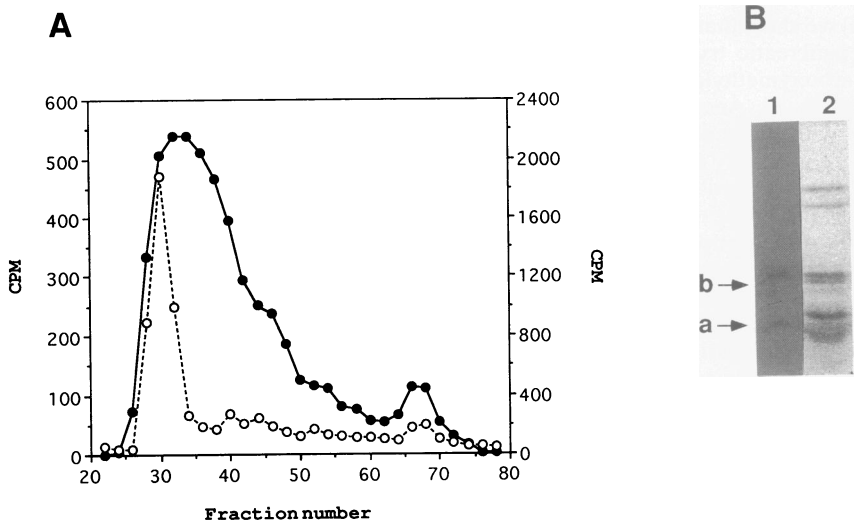

FIG. 4. Separation of overproduced asiA protein from RNA polymerase on Ultrogel AcA 44. (A) RNA polymerase activity and [^{35}S]methionine radioactivity profiles. A Polymin P bacterial extract containing the overproduced asiA protein (1.24×10^6 cpm of trichloroacetic acid-precipitable protein) and RNA polymerase (110 U, with calf thymus DNA as a template) was chromatographed on Ultrogel AcA 44. Details of the procedure are described in Materials and Methods. Five-milliliter fractions were collected. RNA polymerase activity was determined on 50-μl aliquots (right ordinate, open circles), and trichloroacetic acid-precipitable ^{35}S radioactivity was determined on 100-μl aliquots (left ordinate, filled circles). Fractions 66 to 69, containing gp asiA inhibitory activity, were pooled after characterization and stored at −20°C. (B) SDS-polyacrylamide gel analysis of pooled fractions 66 to 69. Lane 1, autoradiography (band a, the radioactive asiA protein; band b, a second, unknown, labeled protein); lane 2, Coomassie brilliant blue staining. Lane 1 was loaded with approximately 10 times the protein amount which is shown in lane 2.

cells. In order to ensure that the inhibitory properties of the partially purified fraction which was to be isolated were indeed due to the overexpressed asiA protein, we ran a parallel series of experiments with JM101 cells carrying the pBSKII (+) plasmid vector.

We reasoned that the asiA protein, once bound to σ^{70}, would tend to dissociate σ^{70} from the core enzyme and that the σ^{70}-asiA protein complex would be separated from RNA polymerase at low ionic strength on the molecular sieve, Ultrogel AcA 44 (21). This expectation turned out to be correct. The experimental fractionation is shown in Fig. 4A. The chromatogram shows an excluded peak containing most of the RNA polymerase activity. This was followed by a shoulder containing few $\alpha_2\beta\beta'$ subunits but large amounts of σ^{70} complexed to radioactive asiA protein. The control column showed a quite different profile, as all the σ^{70} protein was eluted with the RNA polymerase activity in the excluded peak (data not shown).

In the chromatogram of the experimental column (Fig. 4A), we found a small peak with essentially no RNA polymerase activity but showing a very strong RNA polymerase inhibitor activity when the enzyme was assayed with T4 DNA as a template. The fractions of this retarded peak (fractions 66 to 69 [Fig. 4A]) were analyzed by gel electrophoresis and autoradiography. Overproduced gp asiA was a small minority of the low-molecular-weight proteins and accounted for 50% of the radioactivity, as another protein, migrating at the level of reduced and carboxymethylated RNase A, was found labeled (Fig. 4B). In the control column, no trace of RNA polymerase inhibitor activity was detected in the fractions which were eluted after the excluded enzyme peak (data not shown).

The fractions containing the inhibitor activity (Fig. 4A) were dialyzed against a storage buffer containing 50% glycerol and kept at −20°C. The inhibitor activity was stable for more than 6 months. The characteristic saturation profile of the inhibition exerted by gp asiA on the σ-dependent RNA polymerase activity with T4 DNA as a template is shown in Fig. 5. At saturating concentration of inhibitor, the 3 to 5% residual activity corresponds to the low activity of core RNA polymerase acting on T4 DNA (4). The effect of neutral detergents on gp asiA properties was examined. At high

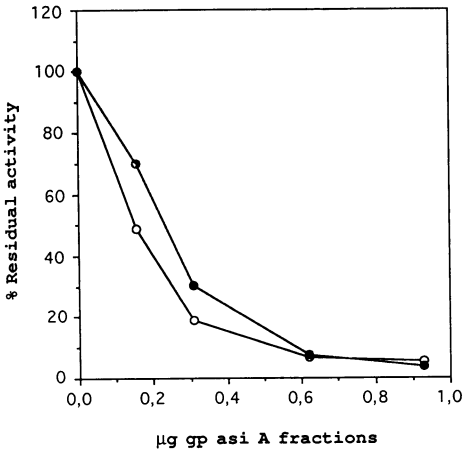

FIG. 5. Inhibition by the overproduced asiA protein of σ-dependent *E. coli* RNA polymerase activity. Inhibition was determined with T4 DNA as a template in the presence of KCl (0.2 M) as a function of increasing amounts of gp asiA contained in fractions 66 to 69 (Fig. 4A). Relative activities are expressed as percentages of maximal enzyme activity. Open circles, partially purified RNA polymerase holoenzyme (specific activity = 64 U/mg; 100% = 1.27 U); filled circles, commercial purified RNA polymerase (Promega Biotec; batch M315) (specific activity = 245 U/mg; 100% = 0.59 U).

TABLE 1. Reversal of gp asiA inhibitory effect of RNA polymerase holoenzyme by Triton X-100 and KCl[a]

Enzyme assay	Activity (U) in the presence of:			
	No additive	KCl	Triton X-100	KCl + Triton X-100
Holoenzyme	1.21	1.48	0.97	1.94
Holoenzyme + gp asiA fractions	0.52	1.18	0.52	1.84

[a] Concentrations: KCl, 0.2 M; Triton X-100, 1%. Overproduced gp asiA (93 ng from pooled fractions 66 to 69 [Fig. 4A]) was used in the inhibition assays.

ionic strength, Triton X-100 antagonizes the inhibitory effect of gp asiA (Table 1). These observations are in accordance with previous results obtained with the detergents Triton X-100 and X-405 (36, 38).

The effect of gp asiA on core RNA polymerase was studied with two different templates. As shown in Table 2, the core enzyme is essentially insensitive to the inhibitor with calf thymus DNA and markedly less sensitive than the holoenzyme with T4 DNA (the 45% inhibition observed with 680 ng of gp asiA fractions on T4 DNA was most likely due to the fact that our core enzyme preparation was not totally free of σ^{70}).

The copolymer poly(dA-dT), whose transcription is independent of σ^{70} activity (5), was used to investigate gp asiA properties relative to the core and holoenzyme. We chose a range of gp asiA concentrations inhibiting 70% of the holoenzyme activity on T4 DNA (Fig. 5). Under these conditions, poly(dA-dT) transcription by holoenzyme showed a 15 to 20% salt-independent inhibition, whereas transcription by core enzyme was totally insensitive to the inhibitor. These results confirm Stevens' previous observations on the 10K protein (36) and show that the inhibitory behavior of gp asiA is dependent on the presence of the σ^{70} subunit in the transcription cycle of *E. coli* RNA polymerase.

DISCUSSION

Over 20 years ago, protein fractions isolated from lysates of bacteria infected with either phage T2 (17) or phage T4 (1, 34) were reported to inhibit the interaction of *E. coli* RNA polymerase core enzyme with its σ subunit. In the latter case, Stevens' careful studies (34–36) established that the T4-dependent anti-σ^{70} activity is borne by a 10-kDa protein, the 10K protein. This protein copurifies with RNA polymerase from T4-infected cells and is detached together with σ^{70} from the core enzyme on phosphocellulose columns (35, 36). These chromatographic properties have been the starting point of the present study. We have partially purified the anti-σ factor and have isolated a tryptic peptide of the protein in order to perform reverse genetics and identify the corresponding gene. Several lines of evidence substantiate our conclusion that gene *asiA* indeed encodes the anti-σ^{70} protein of phage T4.

TABLE 2. Inhibitory effect of gp asiA-containing fractions (Fig. 4A) on core RNA polymerase activity with T4 and calf thymus DNA

gp asiA fractions (μg)	Activity with DNA from[a]:	
	T4 hydroxymethyl-dC	Calf thymus
0	100[b]	100[c]
0.34	98	82
0.68	56	79

[a] Activities are expressed as percentages of the activity for the control and were determined in the presence of 0.2 M KCl.
[b] 100% = 2.15 U.
[c] 100% = 5.70 U.

To partially purify enough of the 10K protein to do peptide sequencing, we have taken advantage of the chromatographic behavior of the 10K protein relative to σ^{70} and to the RNA polymerase core enzyme. We have thus shown (Fig. 1, lanes 3 to 6) that the 10K factor copurifies with σ^{70} flowing through a Bio-Rex 70 column, whereas gp rpbA (45) and gp 55 (28) remain bound to the core enzyme, which is itself retained by this type of column (21).

Gene *asiA* was found to encode a moderately acidic protein with a predicted molecular weight of 10,590. The cloned copy of gene *asiA* showed a high toxicity when we tried to overproduce the asiA protein in *E. coli* BL21(DE3). Once the gene was overexpressed under conditions more suitable for plasmid stability, the overproduced asiA protein was found to modify drastically the interactions of σ^{70} in *E. coli*, in the absence of T4 infection. These modifications provided a basis for the partial purification of the asiA protein. When the protein was overproduced in *E. coli* JM101, σ^{70} was found to be separable from RNA polymerase on a molecular-sieve column and bound to the radioactively labeled asiA protein. This complex was followed by a retarded peak containing some asiA protein free from interaction with σ^{70} and, therefore, appropriate for enzymatic studies (Fig. 4A). A crucial control experiment was carried out by preparing a cell extract from JM101 cells transformed with vector pBluescript II (+) alone, without the inserted gene. When RNA polymerase from this extract was passed through the same column, we found no trace of free σ subunit separated from the excluded holoenzyme peak and, as anticipated, no trace of RNA polymerase inhibitory activity in any of the subsequent fractions.

In the present study, the asiA protein has been only partially purified in order to check its functional properties; despite the partial character of the purification achieved at this stage, the fractions containing the overproduced asiA protein (Fig. 4B) allowed us to repeat essentially all the observations previously reported on the inhibitory properties of the 10K protein coded by phage T4 (36). Minor variations should be noted, however. Stevens has purified the 10K factor by dissociating the complex formed between σ^{70} and 10K in 6 M urea and by passing the dissociated complex through an agarose column equilibrated with the same denaturant (36). The 10K fractions obtained by this method appeared to be electrophoretically homogeneous and devoid of any major contaminating protein. However, this preparation showed a σ-dependent inhibitory effect which, on a molar basis (assuming 100% purity for Stevens' protein and 1 to 5% purity for our protein), appears to be significantly weaker than the one observed under our conditions (Fig. 5) (36). A possible explanation of this quantitative difference is that the 10K protein might have been renatured with a low yield after the 6 M urea chromatography and, hence, was only partially active in the inhibition assays. Our results call for another comment. At high KCl concentration, our RNA polymerase holoenzyme preparation showed a mere 1.22-fold stimulation of its activity with T4 DNA as a template (Table 1). A similar, 1.45-fold factor was observed with a commercial batch of RNA polymerase (mentioned in the legend to Fig. 5). These values are much lower than the 4.9-fold factor reported by Stevens for analogous experi-

ments (36). Enzyme assay conditions most likely account for these differences: we routinely assayed RNA polymerase activity in 100 mM Tris-HCl buffer, instead of the 20 mM used for Stevens' assays, and 20 mM NaCl was added with the T4 hydroxymethyl-dC DNA used as a template. These differences aside and as previously described for the 10K protein (36, 38), at high ionic strength Triton X-100 fully antagonizes the inhibition of RNA polymerase holoenzyme by the asiA protein. The complete insensitivity of RNA polymerase core activity on a poly(dA-dT) template to high concentrations of asiA protein shows quite well that the inhibition of transcription seen with T4 DNA depends on σ^{70} activity.

In a previous report (25), we had identified two regions of the T4 chromosome that coded for proteins which comigrated with the authentic T4 10K protein on one-dimensional SDS-polyacrylamide gels and which bound to RNA polymerase affinity columns with the same avidity as this protein. In two-dimensional polyacrylamide gels, one but not the other of these proteins comigrated with the authentic 10K protein. The DNA fragment which coded for this 10K-like protein was called A* and was defined by a *Pst*I site at 161.35 kb and a *Sal*I site at 20.78 kb on the phage T4 genomic map (19). Gene *asiA* was in the *Sal*I-*Sal*I fragment A of T4 DNA harboring deletion NB5060 but is, in fact, not included in the A* fragment (25). We think that incomplete digestion by *Pst*I would have left the *asiA* gene in a 32.8-kb fragment and this fragment would have been cut out of the agarose gel with the 22.5-kb fragment A*. This could account for this discrepancy. Nonetheless, it is still possible that in the A* fragment there is a gene which codes for a separate, small RNA polymerase-binding protein. The second previously identified 10K RNA polymerase-binding protein coded by fragment G, defined by *Sal*I sites at 111.9 and 143.78 kb, remains to be investigated.

In summary, we have isolated and sequenced gene *asiA* of phage T4 and have presented evidence that its product is identical to the 10K anti-σ^{70} protein described by Stevens many years ago (34–38).

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